

A Rapid-Fire Solution

Introducing the Rapid Response™ Reporter Vectors

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Abstract

To improve the responsiveness of the firefly and Renilla luciferase reporters to rapid changes in transcriptional activity, we have constructed destabilized luciferase reporters containing either protein or protein and mRNA degradation sequences. The half-lives of these Rapid Response™ Reporters are reduced by more than 60%. Consequently, they respond faster and often with greater magnitude to rapid changes in transcriptional rates. As a result, the time required to achieve maximum induction of reporter expression is reduced by up to 75%, which may reduce the risk of artifacts caused by secondary effects.

The Rapid Response™ Reporter Vectors encode destabilized luciferase reporters that rapidly reach maximum induction, increase the ability to discriminate between positive and negative results, and may reduce the risk of secondary effects.

Introduction

Firefly and *Renilla* luciferase reporters are widely used to monitor transcriptional activities in cell biology and drug discovery applications because they are highly sensitive, flexible, and easily quantified. In addition, transcriptional dynamics are revealed quickly due to the relatively low intrinsic stability of the luciferase reporters (protein half-life ~3 hours). However, reporter response still may lag behind the underlying transcriptional events by several hours. To further improve reporter performance, we have developed the Rapid Response™ Reporter Vectors to provide greater expression dynamics. These vectors encode destabilized firefly or *Renilla* luciferase reporters made by genetically fusing either a protein or a protein and mRNA degradation sequence to the luciferase genes. Due to their increased rate of degradation, these destabilized reporters respond faster and often display a greater magnitude of response to rapid transcriptional events.

Increased Response Rate

How does destabilizing a reporter result in increased response rates to rapid transcriptional events? When performing reporter assays, measurements are made on the total accumulation of reporter protein within cells. This accumulation occurs over the intracellular lifetime

of the reporter, which is determined by both protein and mRNA stability. If transcription is changing during this lifetime, then the resulting accumulation of reporter will reflect a collection of different transcriptional rates. The longer the lifetime, the greater the collection of different transcriptional rates pooled into the reporter assay. This pooling process has a “dampening effect” on the representation of transcriptional dynamics, making changes in the transcriptional rate more difficult to detect. This can be remedied by reducing the lifetime of the reporter, thus reducing the pooling of different transcriptional rates into each reporter measurement. The resulting improvement in reporter dynamics is applicable to both upregulation and downregulation of gene expression.

Ideally, the reporter lifetime would be reduced to zero, completely eliminating the pooling of different transcriptional rates in each assay measurement. Only the transcription rate at the instant of the assay would be represented by the accumulation of reporter protein within the cells. Unfortunately, a zero lifetime would also yield zero accumulation, and thus no reporter could be measured. A compromise must be reached since, as lifetime reduces, so does the amount of reporter available for detection in the assay. This is where the high sensitivity of luminescent assays is useful. Relative to other reporter technologies, the intracellular stability of luciferase reporters may be greatly reduced without losing measurable signals. Thus, the high sensitivity of luciferase assays permits greater dynamics in the luciferase reporters.

Design of Destabilized Reporters

During development of the Rapid Response™ Reporters, we evaluated many different protein and mRNA degradation sequences for their effect on response rate and signal magnitude. The best location (i.e., N- or C-terminal) for these degradation sequences was also tested. Two sequences were chosen, one composed of the PEST protein degradation sequence (1) and a second composed of one mRNA (ARE) and two protein (CL1 and PEST) degradation sequences (2–5). The availability of vectors that provide a choice of two configurations of the reporter degradation sequences allows researchers to determine which response rate and corresponding reduction in signal intensity are appropriate for their experimental design.

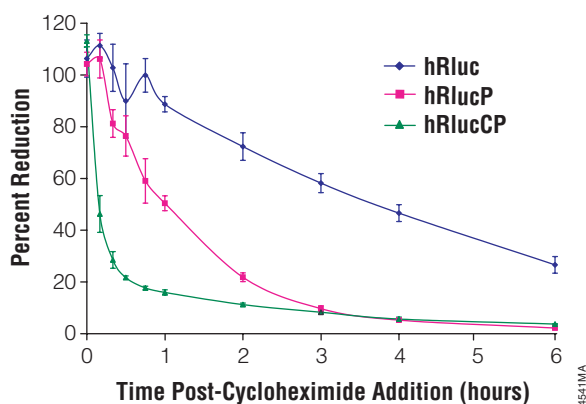


Figure 1. Reduction in reporter half-life for *Renilla*-based Rapid Response™ Reporters. To determine reporter half-life, the Rapid Response™ Reporters and control synthetic *Renilla* luciferase genes were cloned into the pGL3-Control Vector (Cat.# E1741) and transfected into CHO cells. Twenty-four hours post-transfection, cycloheximide (100µg/ml final concentration) was added to each well. At specific time points, cells were collected and the relative light units determined using the *Renilla* Luciferase Assay System (Cat.# E2810).

PEST, which was originally isolated from the C-terminal region of mouse ornithine decarboxylase (1), is a forty-amino acid sequence containing multiple proline (P), glutamate (E), serine (S) and threonine (T) residues flanked by positively charged residues such as lysine (K), arginine (R), or histidine (H). Originally isolated from yeast, the 18-amino acid CL1 sequence has been shown to increase the rate of protein degradation (2). The ARE mRNA degradation sequence is based on the 3'-untranslated region of *Herpesvirus saimiri* small nuclear RNA, which is composed of AU-rich repeats (ARE). AREs are present in the 3'-untranslated regions of many early response genes and have been demonstrated to promote mRNA degradation (3-5).

To increase expression efficiency, the codons in the luciferase reporter genes and protein degradation sequences were optimized for use in mammalian systems. The *hRluc* gene present in the Rapid Response™ Reporter

Vectors is the same as that found in the Promega Synthetic *Renilla* Luciferase Reporter Vectors^(a-e). In addition, to improve reliability of the Rapid Response™ Reporters, most of the known consensus transcription factor binding sites present in the firefly and *Renilla* luciferase reporter genes and in the protein degradation sequences CL1 and PEST were systematically removed. The resulting synthetic reporter genes were designated *hluc+* and *hRluc*, respectively, and the resulting protein degradation sequences were designated hCL1 and hPEST. Because it is not translated, the ARE mRNA degradation sequence was not redesigned.

Half-Life Reduction of Destabilized Reporters

To determine reporter half-lives, the new luciferase genes were cloned into the pGL3-Control Vector^(f,g) (Cat.# E1741) and transfected into CHO cells. Twenty-four hours post-transfection, cycloheximide (100µg/ml final concentration) was added. At specific times, cells were collected and relative light units determined using either Luciferase^(b,h,i) or *Renilla* Luciferase^(a,b) Assay Systems. Inclusion of the PEST degradation sequence reduced the half-life for *hRlucP* by more than 60% from 3 hours to approximately 1 hour. The addition of CL1, PEST and ARE had the greatest effect on half-life; the half-life of *hRlucCP* was reduced more than 80% to 0.4 hours (Figure 1). Similar results were obtained with the firefly luciferase-based Rapid Response™ Reporters (data not shown).

Reduced Time and Greater Magnitude of Response

Four Rapid Response™ Reporter Vectors are available, two encoding destabilized firefly luciferases and two encoding destabilized *Renilla* luciferases (Figure 2). The pGL3(R2.1)-Basic and pGL3(R2.2)-Basic Vectors^(a,b,c,g), encode firefly luciferase and contain the *hlucP+* and *hlucCP+* genes, respectively. Similarly, the phRG(R2.1)-Basic and phRG(R2.2)-Basic Vectors^(a-d) encode *Renilla* luciferase and contain the *hRlucP* and *hRlucCP* genes, respectively (Figure 2).

Increased Protein Degradation

Vector	Reporter Gene	Gene Design
phRG(R2.1)-Basic	<i>hRlucP</i>	
pGL3(R2.1)-Basic	<i>hlucP+</i>	

Increased Protein and mRNA Degradation

Vector	Reporter Gene	Gene Design
phRG(R2.2)-Basic	<i>hRlucCP</i>	
pGL3(R2.2)-Basic	<i>hlucCP+</i>	

Figure 2. Schematic representation of the Rapid Response™ reporter genes.

Rapid Response™ Reporter Vectors... continued

To determine whether reducing reporter stability also reduced the time required for maximum response, a DNA fragment containing multiple cAMP response elements (CREs) was cloned into the Rapid Response™ Vectors and transfected into HEK293 cells. The cells were induced with isoproterenol hydrochloride (ISO), an agonist for endogenous adrenergic receptors, and luciferase expression was measured. The time for maximum induction of reporter expression for the destabilized *Renilla* reporters, hRlucP and hRlucCP, was reduced compared to that of the hRluc control. The hRluc control reached maximum induction at 8 hours, while maximum induction for hRlucCP and hRlucP occurred at 3.0 and 4.5 hours, respectively (Figure 3, Panel A).

The time needed for maximum induction of the destabilized firefly luciferase reporters was similarly reduced. Maximum induction for the control firefly luciferase (hluc+) was reached in 6 hours, while the destabilized reporters, hlucCP+ and hlucP+, reached maximum induction in 1.5 and 3 hours, respectively (Figure 3, Panel B).

In addition to reducing the time to maximum induction, the magnitude of the response was also greater for the destabilized reporters. The *Renilla*-based Rapid Response™ Reporters (Figure 3, Panel A) display greater than 67% increase in magnitude of response. For the firefly luciferase-based Rapid Response™ Reporters hlucP+ and hlucCP+, the response was increased by greater than 102% (Figure 3, Panel B).

Because the Rapid Response™ reporters are destabilized, light intensity in the reporter assay is reduced (Figure 4). This reduction varies based on the cell line and destabilized reporter used, and on whether stably or transiently transfected cells are used. For stably expressing cell lines, some of the brightest cell lines isolated (i.e., those with the highest relative light units per cell) were expressing the Rapid Response™ Reporters (data not shown). Similar results have been observed for other destabilized reporters (6,7). Whether a reduction in light intensity is significant should be evaluated in the context of assay sensitivity. Firefly and *Renilla* luminescent assays generate very low background signal, thus the sensitivity of the Rapid Response™ Reporters is generally sufficient for most experimental conditions.

Pharmacological Equivalency

To address whether the Rapid Response™ Reporters display similar responses to physiological conditions as their non-destabilized equivalents, vectors containing CRE, *neo*, and either *hluc+*, *hlucP+* or *hlucCP+* were transfected into HEK293 cells. After neomycin selection, clones stably expressing luciferase were isolated. The cells were incubated with increasing concentrations of ISO and tested for luciferase activity at 2, 4, and 6 hours post-ISO addition (Figure 5). Data from the earliest time point (2 hours) for *hlucP+* and *hlucCP+* are highly similar to the data from the 6-hour time point for *hluc+*. The similarity of these ISO dose-response curves demonstrates equivalent pharmacological responses from the cells.

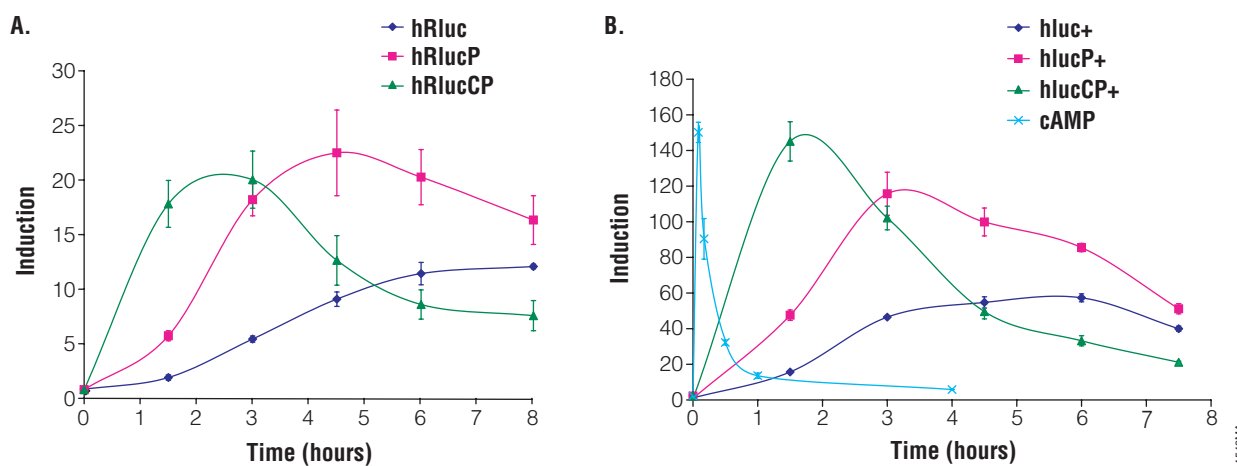


Figure 3. Time to achieve maximum induction for the Rapid Response™ Reporters. A DNA segment containing multiple CREs (cAMP Response Elements) was cloned into each of the four Rapid Response™ Reporter Vectors and into the pGL3-Basic Vector⁽¹⁹⁾ (Cat.# E1751) containing either the *hluc+* or *hRluc* reporter genes. The CRE-containing vectors were transiently transfected into HEK293 cells. After 24 hours, 100 μ M of RO (RO-20-1724) and 1 μ M isoproterenol hydrochloride (ISO) were added to induce reporter gene expression. RO alone (100 μ M) was added to a subset of the wells to serve as a noninduced control. Cells were harvested, lysed and assayed as follows: **Panel A.** Cells containing *Renilla*-based reporters were assayed for luciferase activity using the *Renilla* Luciferase Assay System (Cat.# E2810). **Panel B.** Cells containing firefly-based luciferase reporters were assayed for luciferase activity using the Luciferase Assay System (Cat.# E1500), and for cAMP using the Correlate-EIA Directed Cyclic AMP Assay (Assay Designs, Inc.). In all cases, induction was calculated by dividing the relative light units obtained from induced wells by the relative light units obtained from noninduced wells.

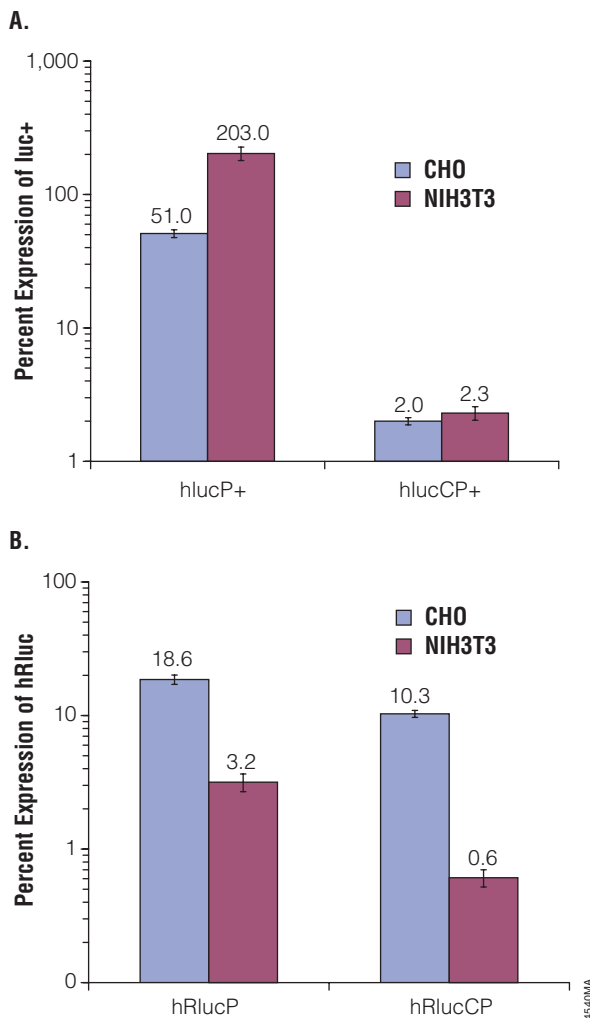


Figure 4. Expression of Rapid Response™ Reporters in CHO and NIH3T3 cells. The *luc+* gene present in the pGL3-Control Vector (Cat.# E1741) was replaced with either *hLucP+*, *hLucCP+*, *hRluc*, *hRlucP* or *hRlucCP* reporter genes. The resulting vectors and the pGL3-Control Vector were cotransfected with a second reporter (transfection control) into either CHO or NIH3T3 cells. The transfection controls for Panels A and B were phRL-TK^(e-d) (Cat.# E6241) and pGL3-Control (Cat.# E1741) Vectors, respectively. Twenty-four hours post-transfection the cells were harvested with Passive Lysis Buffer (Cat.# E1941), and relative light units were determined using the Dual-Luciferase® Assay System^(b,h,i) (Cat.# E1910). The relative light units were normalized to the transfection control. The effects of the degradation sequences on the accumulation of luciferase enzyme are shown as percent of the control. **Panel A.** Percent expression of hLucP+ and hLucCP+ versus luc+ (pGL3-Control Vector) in CHO and NIH3T3 cell transfections. **Panel B.** Percent expression of hRlucP and hRlucCP versus hRluc in CHO and NIH3T3 cell transfections.

Thus, by destabilizing the luciferase reporters, we have reduced the time to reach maximum induction, increased the ability to discriminate between positive and negative results, and reduced the risk of secondary effects. Yet, in this experiment, the interrelationship between transcriptional rates and cellular physiology events remained unaltered.

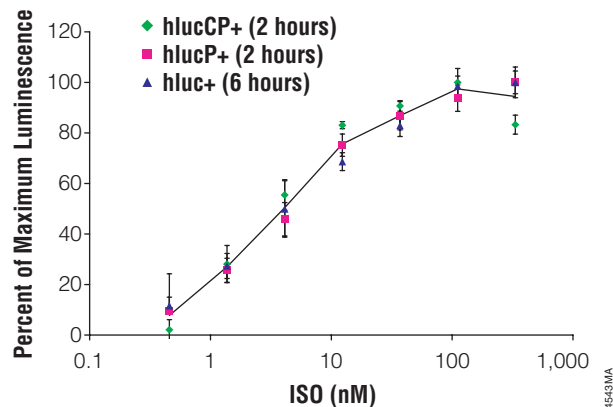


Figure 5. Pharmacological agonist profiles for the Rapid Response™ Reporters. Vectors containing CRE, *neo* and either *hLuc+*, *hLucP+*, or *hLucCP+* were used to create stably expressing HEK293 cells. Cells were seeded into 96-well plates and incubated with various concentrations of isoproterenol hydrochloride (ISO) for 2, 4 and 6 hours. Control wells were untreated. After incubation, the Bright-Glo™ Luciferase Assay System^(b,h,i) (Cat.# E2610) was used to measure luciferase activity. The line represents the mean of all 3 data sets.

Conclusion

The Rapid Response™ Reporter technology is composed of firefly and *Renilla* luciferase reporters that have been destabilized by the addition of protein or protein and mRNA degradation sequences. These destabilized reporters respond faster and with greater magnitude to changes in transcriptional rate. The faster response rate of the Rapid Response™ Reporters allows better coupling of their response to transient cellular events, which may reduce the risk of secondary events interfering with experiment results. The faster response rate can also enable higher assay throughput in screening applications. These improvements do not affect the underlying physiological conditions coupled to the reporter expression.

References

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Protocol

- ◆ *Rapid Response™ Reporter Vectors Technical Manual #TM242*, Promega Corporation. (www.promega.com/tbs/tm242/tm242.html)

Rapid Response™ Reporter Vectors... continued

Ordering Information

Product	Size	Cat.#
pGL3(R2.1)-Basic Vector ^(a,b,c,g)	20µg	E6431
pGL3(R2.2)-Basic Vector ^(a,b,c,g)	20µg	E6441
phRG(R2.1)-Basic Vector ^(a-d)	20µg	E6451
phRG(R2.2)-Basic Vector ^(a-d)	20µg	E6461

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- ^(f) U.S. Pat. No. 5,670,356.
- ^(g) The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.
- ^(h) U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289 and 5,814,471, Australian Pat. No. 649289 and other patents and patents pending.
- ⁽ⁱ⁾ The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.
- ^(j) U.S. Pat. No. 5,744,320, Australian Pat. No. 721172, Canadian Pat. No. 2,221,522 and other patents and patents pending.

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